

AD \_\_\_\_\_

Award Number: DAMD17-02-C-0106

TITLE: Combinatorial Strategies and High Throughput Screening  
in Drug Discovery Targeted to Channel of Botulinum

PRINCIPAL INVESTIGATOR: Mauricio Montal, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of California - San Diego  
La Jolla, California 92093-0934

REPORT DATE: September 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20050105 052

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY</b> (Leave blank)		<b>2. REPORT DATE</b> September 2004		<b>3. REPORT TYPE AND DATES COVERED</b> Annual (1 Sep 03 - 31 Aug 04)	
<b>4. TITLE AND SUBTITLE</b> Combinatorial Strategies and High Throughput Screening in Drug Discovery Targeted to Channel of Botulinum				<b>5. FUNDING NUMBERS</b> DAMD17-02-C-0106	
<b>6. AUTHOR(S)</b> Mauricio Montal, M.D., Ph.D.					
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> University of California - San Diego La Jolla, California 92093-0934  E-Mail: mmontal@ucsd.edu				<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>					
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited				<b>12b. DISTRIBUTION CODE</b>	
<b>13. ABSTRACT (Maximum 200 Words)</b> <p>The ultimate goal of this program is to discover selective and potent drugs targeted to prevent or relieve the neurotoxic actions of botulinum neurotoxin (BoNT) A. A major goal of this program is the identification of open channel blockers as a single class of drugs that would be effective against all BoNT isoforms. The major focus thus far has been the implementation of a reliable and robust high-throughput screen for blockers specific for BoNT. This facet of the program involves the use of the VIPR™ -Voltage/Ion Probe Reader, a proven strategy for high-throughput screening, using nerve growth factor-differentiated pheochromocytoma PC12 cells in which BoNTA forms channels with similar properties to those previously characterized in lipid bilayers. The fidelity of the assay relies on fluorescence measurements of membrane potential changes as an index of open BoNT channels and increased cation conductance. The immediate task is to select mixtures from synthetic combinatorial libraries with high blocking activity to deconvolute and identify the most potent compounds. We consider the BoNT channel as a validated target for intervention aimed to inhibit the translocation of the light chain into the cytosol and therefore to attenuate the BoNT neurotoxicity.</p>					
<b>14. SUBJECT TERMS</b> Botulinum neurotoxin; drug discovery; ion channels; channel blockers; combinatorial chemistry				<b>15. NUMBER OF PAGES</b> 17	
				<b>16. PRICE CODE</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited		

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18  
298-102

## Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4
Conclusions.....	6
Key Research Accomplishments.....	6
Reportable Outcomes.....	6
References.....	7
Appendices.....	7
Figures .....	8
Appendix #1 .....	11

## INTRODUCTION

This program examines innovative approaches and powerful new technologies to identify selective and potent agents directed to prevent or relieve the neuroparalytic toxic actions of botulinum toxin A (BoNTA)<sup>1</sup>. The focus is on the ion channel forming activity of BoNTs as a validated target to screen for inhibitors of the translocation of the light chain into the cytosol and therefore to attenuate the BoNT neurotoxicity. The key of the program is based on our discovery that the heavy chain (HC) of BoNT acts as both a channel and a transmembrane chaperone for the light chain (LC) to ensure a translocation competent conformation during its transit from the acidic endosome into the cytosol - its site of action<sup>2</sup>. This is an exciting time to focus on innovative technologies to uncover lead compounds that may represent a potential new generation of useful and safe antidotes for BoNTs.

Two major aims, representing different technologies, are pursued:

- Task 1: Use of synthetic combinatorial libraries (SCL)<sup>3</sup> to discover specific inhibitors of the channel activity of BoNTs.
- Task 2: Use of the VIPR™ -Voltage/Ion Probe Reader<sup>4</sup>, for high-throughput screening (HTS) for open channel blockers of BoNTA channel on Neuro 2A cells.

Evidently, the two tasks are intricately connected. However, inhibitor discovery requires the implementation of a robust and reliable HTS. This has been the main focus of the program during the first facet of this project as reported in the last Annual Report. Here, we focus on the progress concerning the implementation of a neuronal system amenable to characterize the channel and chaperone activities of BoNT under conditions which closely emulate those prevalent at the endosome, and which are relevant to the neurotropism and neuroparalytic effects of BoNTs.

## BODY

The channel and chaperone activities of *Clostridial botulinum* neurotoxin (BoNT) A were investigated in Neuro 2A neuroblastoma cells under conditions that closely emulate those prevalent at the endosome. Neuro 2A neuroblastoma cells, derived from a spontaneous tumor of a Strain A albino mouse, constitute a good neuronal model for neurosecretion and electrophysiology. Neuro 2A cells are highly sensitive to BoNTs<sup>5</sup> and are readily accessible for patch clamp recordings<sup>6</sup>. For this purpose, the patch pipets contain BoNT and the solution is buffered at pH 5.3 while the bath solution is buffered at pH 7.0. Neuro 2A cells are plated onto Matrigel coated glass coverslips at 500 cells/coverslip, and cultured at 37°C, 5% CO<sub>2</sub> for 1-3 days prior to patch clamp recordings. To attenuate or eliminate endogenous channel activity while selectively augmenting the detection of BoNT channel currents, the external and internal solutions contain CsCl: Cs<sup>+</sup> acts as the current carrier for the BoNT channel<sup>7</sup> and it does

not permeate through endogenous  $K^+$  or  $Na^+$  channels<sup>6</sup>. The external (bath) solution contains (in mM) CsCl 200, 3-(N-morpholino) propanesulfonic acid 5, DTT (dithiothreitol) 1, (pH 7.0 with HCl), and the internal (pipet) solution contains (in mM) CsCl 200, DTT 1, 2-(N-morpholino) ethanesulfonic acid 5, (pH 5.3 with HCl). Channel insertion is achieved by supplementing 5  $\mu$ g/mL BoNTA holotoxin to the internal (pipet) solution, which is set to an endosomal pH of 5.3. After gigaohm ( $G\Omega$ ) seal formation, the patch is excised from the cell and current recordings are obtained under voltage clamp conditions.

## Results

Toxin insertion and channel formation are pH dependent; no channels are detected when the internal solution is held at pH 7 rather than pH 5.3. The current flowing through individual BoNT channels at the indicated voltages is shown in *Figure 1*. The channels are voltage dependent opening only at negative voltages. Single-channel currents were determined at each voltage from amplitude histograms<sup>8</sup>. *Figure 2* displays the single-channel current ( $I$ ) –voltage ( $V$ ) curves for BoNT channels. BoNT channels open to a main conductance of  $\sim 90$  pS (*Fig. 2a*) and display a conspicuous subconductance of  $\sim 10$  pS (*Fig. 2b*). Inspection of *Figure 1* clearly shows that the BoNT channel activity occurs in bursts interspersed between periods of little or no activity; the frequency of burst occurrence is also voltage dependent, increasing with negative voltages up to  $\sim -80$  mV. The quiescent periods between bursts are prolonged at voltages more negative than this threshold even though the channel fast gating to the open state continues to increase up to  $-110$  mV.

Within the bursts, the channel resides preferentially in the open state (O) making frequent and fast transitions to the closed state (C). The open channel probability ( $P_o$ ) within bursts sharply increases with negative voltages: the voltage at which  $P_o = 0.5$  is  $-30 \pm 6$  mV (*Fig. 2d*).

At the onset of a burst, the channel enters the 10 pS subconductance state (S) and then undergoes quick transitions between this intermediate state and the open state. This is clearly shown in *Figure 3*. The open state shows very fast transitions to the substate and to the closed state giving the appearance of flickering between the open state and the substate, the predominant activity within a burst. A section delimited by the arrows is displayed at higher time resolution in the lower panel, where transitions between the three indicated states are clearly discerned. Channel opening (O) is indicated as a downward deflection and the approximate value of the subconductance state (S) is marked. The characteristic opening and closing transitions are resolved, showing the occurrence of fast transitions to the substate. *Figure 2c* illustrates the transitions between a closed (C), open substate (S) and the fully open state (O). The magnitude of the arrows connecting the states is proportional to the frequency of occurrence of transitions between the three states. Rarely within a burst the channel returns to the closed state from the open state, exhibiting a high propensity to fluctuate between the subconductance and open states or the subconductance and closed states. Thus, the subconductance state and the transitions into and out of this state are clearly recognized in the single-channel

recordings. This pattern of channel activity was previously identified in single-channel recordings from tetanus toxin reconstituted in lipid bilayers<sup>9</sup>, suggesting similarities of the assembled and functional clostridial channels in membranes. The relationship between the subconductance state and the translocation events is, at present, undetermined given that the subconductance state is also exhibited by the heavy chain channel in the absence of the light chain. Work in progress is directed to assess the role of the heavy chain "belt" in the occurrence of substates. Constructs are currently in design to produce a "beltless" heavy chain; the channel activity will be characterized under identical conditions as those used for the wild type holotoxin and heavy chain.

## CONCLUSIONS

A salient feature of the BoNT channel is that it is closed at positive voltages under conditions in which the orientation and the magnitude of the pH gradient, as well as the polarity and magnitude of the membrane potential compare fairly well with those prevailing across the endosomal membrane: pH 5.3 and positive potential on the compartment containing the BoNT and pH 7.0 and negative potential on the opposite compartment. This suggests that the BoNT heavy chain channel would be closed in the endosome until it is gated by the BoNT light chain to initiate its translocation across the membrane into the cytosol. The neuroblastoma cell line appears, therefore, to be a suitable system to characterize the BoNT channel and to pursue evaluation of plausible strategies for targeted drug delivery thereby minimizing the requirement for *in vivo* animal testing.

## KEY RESEARCH ACCOMPLISHMENTS

- A key step in the intoxication by BoNT is the translocation of internalized toxin across intracellular membranes to reach its cytosolic targets. A fundamental discovery was the demonstration that the heavy chain acts as both a channel and a transmembrane chaperone for the light chain protease to ensure a translocation competent conformation during transit from acidic endosomes into the cytosol. Thus, the stage is set for pursuing the identification of channel blockers specific for the BoNT heavy chain.
- A high-throughput screen exploiting the VIPR™-Voltage/Ion Probe Reader, is now a proven strategy for the search of compounds that modulate or block the channel activity of BoNT on Neuro 2A cells.

## REPORTABLE OUTCOMES

- Publications

Appendix # 1: Fischer, A. and Montal, M. Characterization of *Clostridial botulinum* neurotoxin channels in neuroblastoma cells. Bioscience Review 2004

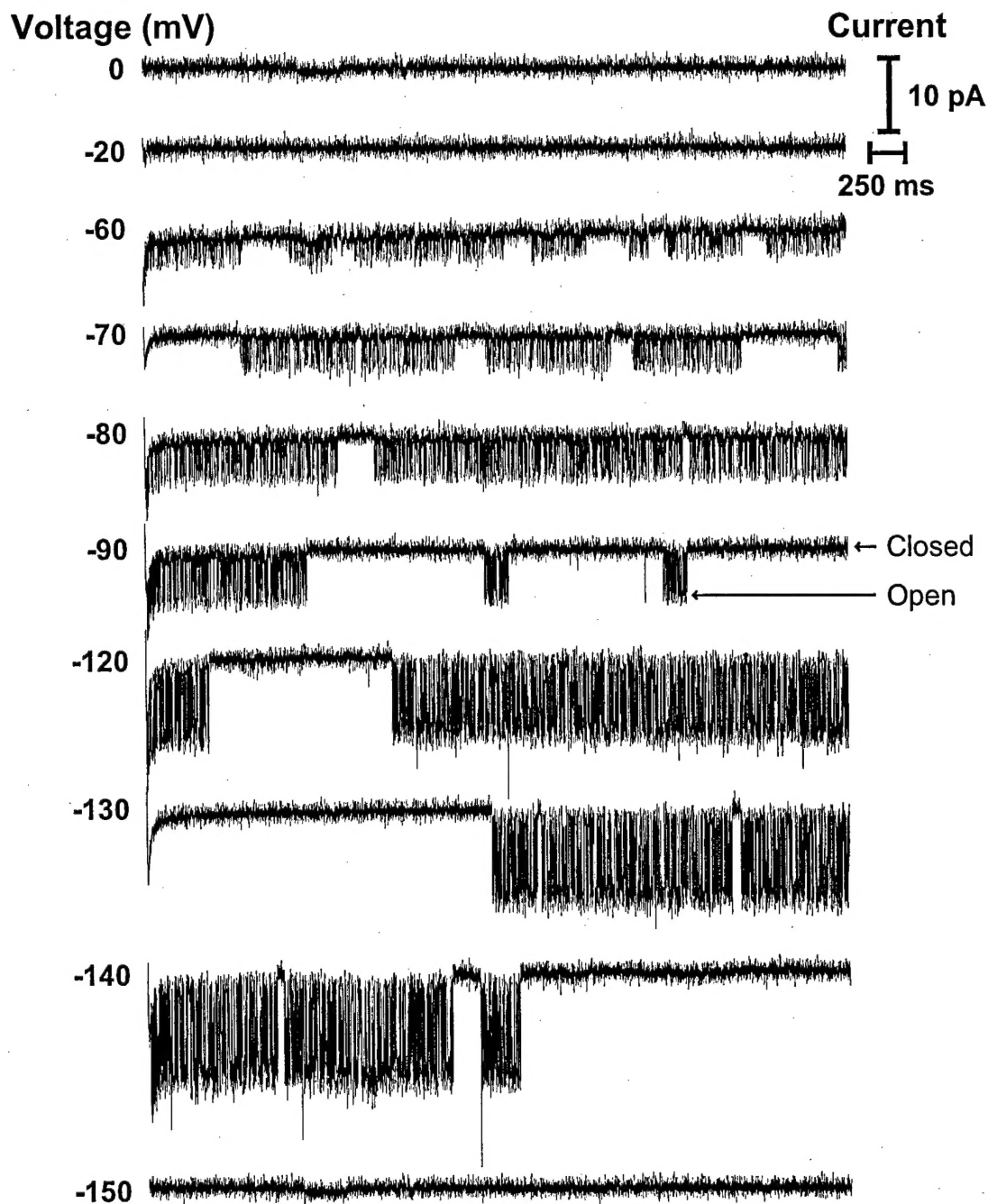
## REFERENCES

1. Schiavo, G., Matteoli, M. & Montecucco, C. Neurotoxins affecting neuroexocytosis. *Physiological Reviews* **80**, 717-66 (2000).
2. Koriazova, L. K. & Montal, M. Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. *Nat Struct Biol* **10**, 13-8 (2003).
3. Tai, K. K., Blondelle, S. E., Ostresh, J. M., Houghten, R. A. & Montal, M. An N-methyl-D-aspartate receptor channel blocker with neuroprotective activity. *Proc Natl Acad Sci U S A* **98**, 3519-24 (2001).
4. Gonzalez, J. E. & Maher, M. P. Cellular fluorescent indicators and voltage/ion probe reader (VIPR) tools for ion channel and receptor drug discovery. *Receptors Channels* **8**, 283-95 (2002).
5. Yowler, B. C., Kensinger, R. D. & Schengrund, C. L. Botulinum neurotoxin A activity is dependent upon the presence of specific gangliosides in neuroblastoma cells expressing synaptotagmin I. *J Biol Chem* **277**, 32815-9 (2002).
6. Hille, B. *Ion Channels of Excitable Cells* (Sinauer, Sunderland, MA, 2001).
7. Sheridan, R. E. Gating and permeability of ion channels produced by botulinum toxin types A and E in PC12 cell membranes. *Toxicon* **36**, 703-17. (1998).
8. Keller, B. U., Hartshorne, R. P., Talvenheimo, J. A., Catterall, W. A. & Montal, M. Sodium channels in planar lipid bilayers. Channel gating kinetics of purified sodium channels modified by batrachotoxin. *J Gen Physiol* **88**, 1-23. (1986).
9. Gambale, F. & Montal, M. Characterization of the channel properties of tetanus toxin in planar lipid bilayers. *Biophys J* **53**, 771-83. (1988).

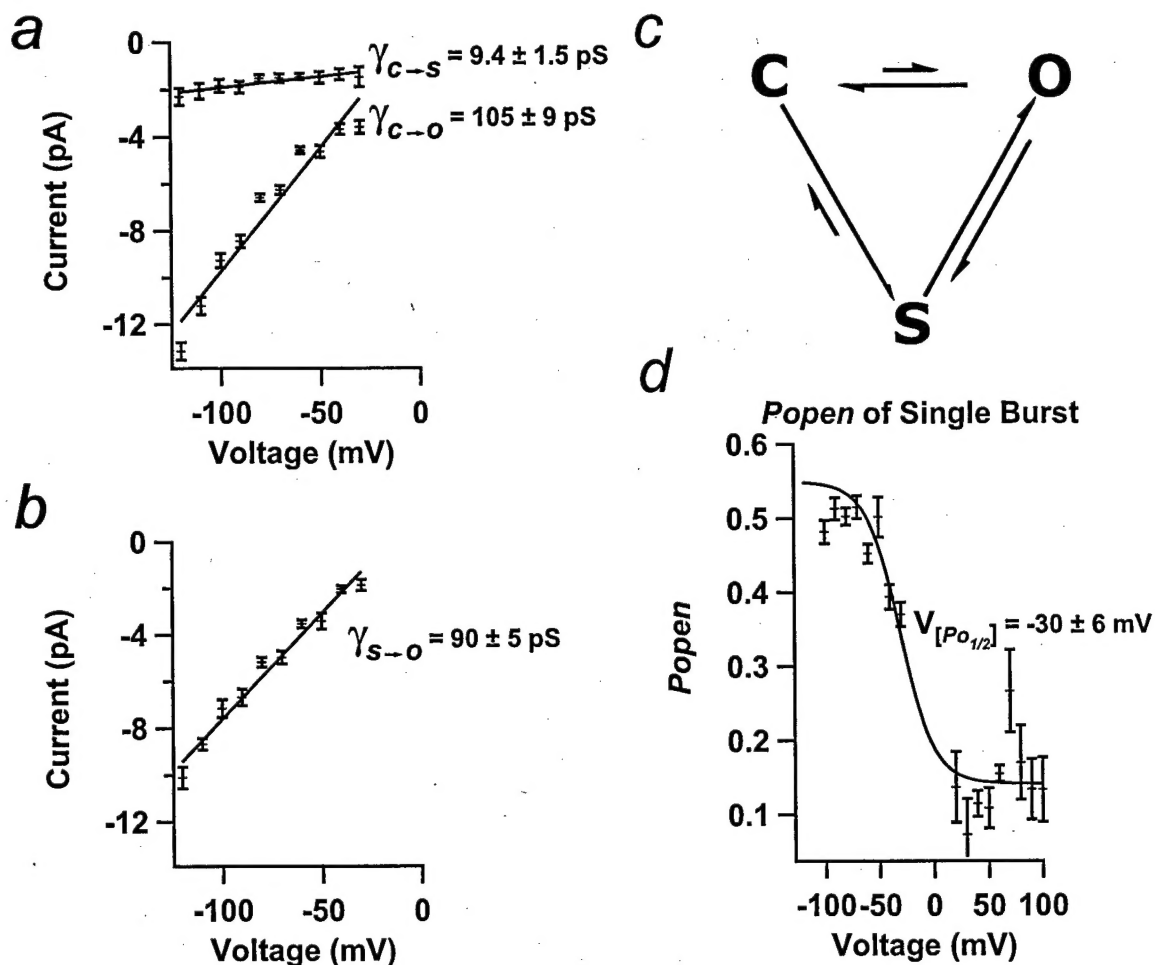
## APPENDICES

### Appendix #1

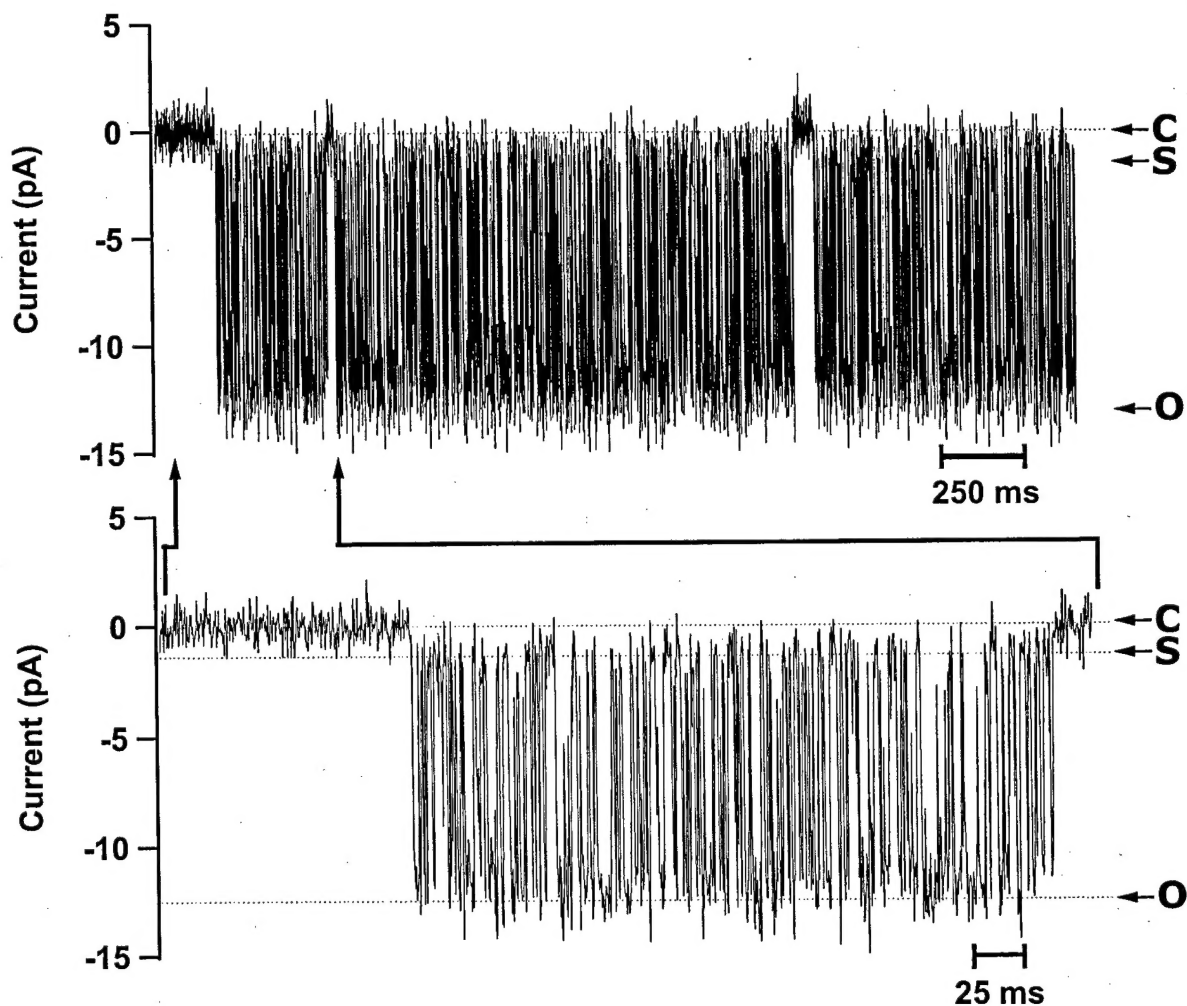
Fischer, A. and Montal, M. Characterization of *Clostridial botulinum* neurotoxin channels in neuroblastoma cells. Bioscience Review 2004 (in press).



**Figure 1.** Botulinum neurotoxin bursting channel activity in excised patches of Neuro 2A cells. Representative single-channel currents at the indicated voltages. Consecutive stimulations on the same patch. Channel opening is indicated by a downward deflection. BoNT A channels open to a main conductance of  $\sim 90$  pS.



**Figure 2.** Analysis of BoNT A channels in Neuro 2A cells. a, Single channel current-voltage characteristics for transitions of the closed state (C) to substate (S) and closed state to open state (O). b, Single channel current-voltage characteristics for transitions of the substate to the open state. c, Transitions between a closed (C), substate (S) and fully open (O) state of BoNT A channels. Arrow length is proportional to probability of transition. d, Probability of channel residence in the open state as a function of voltage. All analysis based upon single bursts of channel activity within a record ( $N \geq 6$ ).



**Figure 3.** BoNT A bursting channel activity in excised patches of Neuro 2A cells. At -130 mV transitions from the closed (C) to the open (O) state and from the substate (S) to the open state are clearly resolved. The BoNT A channels open to a main conductance of ~90 pS and a subconductance of ~10 pS. The approximate value for the subconductance state (S) is marked. The lower panel shows the section of the record delimited by the arrows at higher time resolution.

## APPENDIX 1

Characterization of *Clostridial botulinum* neurotoxin channels in neuroblastoma cells.

Audrey Fischer and **Mauricio Montal**. Section of Neurobiology, Division of Biological Sciences, University of California San Diego, La Jolla, CA 92093-0366.

### Abstract

The channel and chaperone activities of *Clostridial botulinum* neurotoxin (BoNT) A were investigated in Neuro 2A neuroblastoma cells under conditions that closely emulate those prevalent at the endosome. Channel activity occurs in bursts interspersed between periods of little or no activity. The channels are voltage dependent, opening only at negative voltages to a main conductance of ~90 pS and display a conspicuous subconductance of ~10 pS. Within bursts, the channel resides preferentially in the open state. The neuroblastoma cell line appears, therefore, to be a suitable system to characterize the BoNT channel and to pursue evaluation of plausible strategies for targeted drug delivery thereby minimizing the requirement for *in vivo* animal testing.

Keywords: botulinum neurotoxin; channels; chaperones; targeted drug screen

This work was supported by the U.S. Army Medical Research and Materiel Command under Contract/Grant/Intergovernment Project Order DAMD17-02-C-0106.

## Introduction

Chemoprophylaxis and therapeutic intervention of the deadly intoxication by botulinum neurotoxins (BoNT) requires understanding the mechanisms by which it abrogates neurotransmitter release. Our aim is to elucidate the fundamental molecular mechanisms that confer to BoNT its exquisite ability to move efficiently and selectively within neurons exploiting its modular organization. An immediate objective is to understand the intricacies of intracellular trafficking of BoNT that may disclose novel pathways to escort, target, and insert it into membranes. This information may prove diagnostic in identifying unsuspected sites for intervention and plausible strategies for targeted drug delivery.

The key of the program is based on our discovery that the heavy chain of BoNT acts as both a channel and a transmembrane chaperone for the light chain to ensure a translocation competent conformation during its transit from the acidic endosome into the cytosol – its site of action<sup>2</sup>. The light chain is a  $\text{Zn}^{2+}$ -metalloprotease that cleaves the protein components involved in synaptic vesicle fusion with the neuronal membrane, thereby abrogating synaptic transmission<sup>1</sup>. To accomplish this task, the heavy chain operates as a transmembrane protein-conducting channel: the channel is occluded by the light chain during transit, and open after completion of translocation and release of cargo, acting intriguingly similar to the protein-conducting/translocating channels of the endoplasmic reticulum (ER), mitochondria, and chloroplasts. This finding has outlined a novel way of thinking about BoNT neurotoxicity shifting the focus of attention on its translocation within cells rather than on the protease activity of the light chain, which is known not to be toxic unless it is internalized. A major goal of this program is the identification of open channel blockers as a single class of drugs that would be effective against all seven *Clostridial botulinum* neurotoxin isoforms. To accomplish this task we have developed a neuronal system amenable to characterize the channel and chaperone activities of BoNT under conditions which closely emulate those prevalent at the endosome, and which are relevant to the neurotropism and neuromuscular effects of BoNTs.

## Methods

Neuro 2A neuroblastoma cells, derived from a spontaneous tumor of a Strain A albino mouse, constitute a good neuronal model for neurosecretion and electrophysiology. Neuro 2A cells are highly sensitive to BoNTs<sup>5</sup> and are readily accessible for patch clamp recordings<sup>6</sup>. For this purpose, the patch pipets contain BoNT and the solution is buffered at pH 5.3 while the bath solution is buffered at pH 7.0. Neuro 2A cells are plated onto Matrigel coated glass coverslips at 500 cells/coverslip, and cultured at 37°C, 5%  $\text{CO}_2$  for 1-3 days prior to patch clamp recordings. To attenuate or eliminate endogenous channel activity while selectively augmenting the detection of BoNT channel currents, the external and internal solutions contain CsCl:  $\text{Cs}^+$  acts as the current carrier for the BoNT channel<sup>7</sup> and it does not permeate through endogenous  $\text{K}^+$  or  $\text{Na}^+$  channels<sup>6</sup>. The external (bath) solution contains (in mM) CsCl 200, 3-(N-morpholino) propanesulfonic acid 5, DTT (dithiothreitol) 1, (pH 7.0 with HCl), and the internal (pipet) solution contains (in mM) CsCl 200, DTT 1, 2-(N-morpholino) ethanesulfonic acid 5, (pH 5.3 with HCl). Channel insertion is achieved by supplementing 5  $\mu\text{g}/\text{mL}$  BoNTA holotoxin to the internal (pipet) solution, which is set to an endosomal

pH of 5.3. After gigaohm ( $G\Omega$ ) seal formation, the patch is excised from the cell and current recordings are obtained under voltage clamp conditions.

### Results

Toxin insertion and channel formation are pH dependent; no channels are detected when the internal solution is held at pH 7 rather than pH 5.3. The current flowing through individual BoNT channels at the indicated voltages is shown in *Figure 1*. The channels are voltage dependent opening only at negative voltages. Single-channel currents were determined at each voltage from amplitude histograms<sup>8</sup>. *Figure 2* displays the single-channel current ( $I$ ) –voltage ( $V$ ) curves for BoNT channels. BoNT channels open to a main conductance of  $\sim 90$  pS (*Fig. 2a*) and display a conspicuous subconductance of  $\sim 10$  pS (*Fig. 2b*). Inspection of *Figure 1* clearly shows that the BoNT channel activity occurs in bursts interspersed between periods of little or no activity; the frequency of burst occurrence is also voltage dependent, increasing with negative voltages up to  $\sim -80$  mV. The quiescent periods between bursts are prolonged at voltages more negative than this threshold even though the channel fast gating to the open state continues to increase up to  $-110$  mV.

Within the bursts, the channel resides preferentially in the open state (O) making frequent and fast transitions to the closed state (C). The open channel probability ( $P_o$ ) within bursts sharply increases with negative voltages: the voltage at which  $P_o = 0.5$  is  $-30 \pm 6$  mV (*Fig. 2d*).

At the onset of a burst, the channel enters the 10 pS subconductance state (S) and then undergoes quick transitions between this intermediate state and the open state. This is clearly shown in *Figure 3*. The open state shows very fast transitions to the substate and to the closed state giving the appearance of flickering between the open state and the substate, the predominant activity within a burst. A section delimited by the arrows is displayed at higher time resolution in the lower panel, where transitions between the three indicated states are clearly discerned. Channel opening (O) is indicated as a downward deflection and the approximate value of the subconductance state (S) is marked. The characteristic opening and closing transitions are resolved, showing the occurrence of fast transitions to the substate. *Figure 2c* illustrates the transitions between a closed (C), open substate (S) and the fully open state (O). The magnitude of the arrows connecting the states is proportional to the frequency of occurrence of transitions between the three states. Rarely within a burst the channel returns to the closed state from the open state, exhibiting a high propensity to fluctuate between the subconductance and open states or the subconductance and closed states. Thus, the subconductance state and the transitions into and out of this state are clearly recognized in the single-channel recordings. This pattern of channel activity was previously identified in single-channel recordings from tetanus toxin reconstituted in lipid bilayers<sup>9</sup>, suggesting similarities of the assembled and functional clostridial channels in membranes.

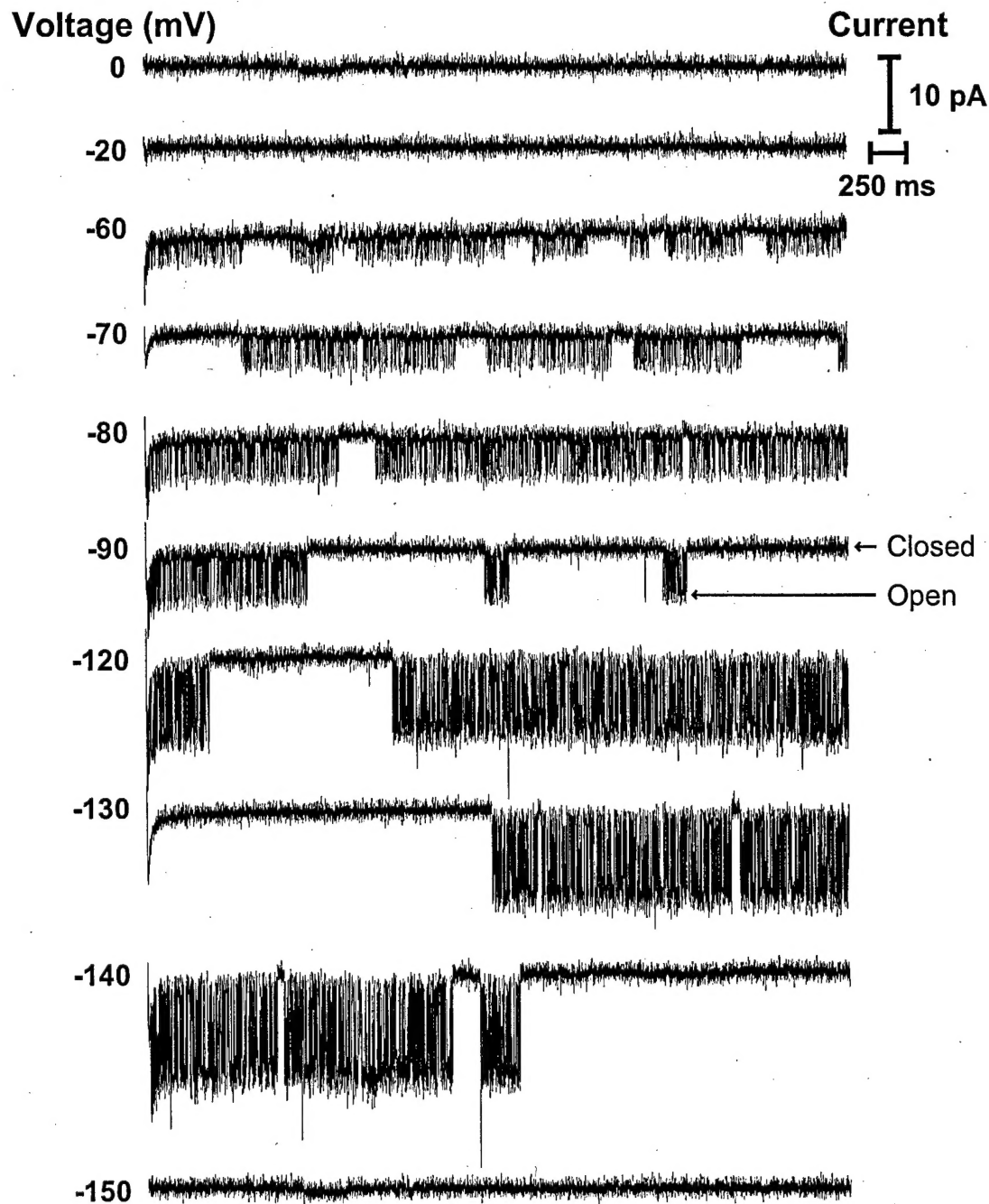
### Conclusions

A salient feature of the BoNT channel is that it is closed at positive voltages under conditions in which the orientation and the magnitude of the pH gradient, as well as the polarity and magnitude of the membrane potential compare fairly well with those prevailing across the endosomal membrane: pH 5.3 and positive potential on the compartment containing the BoNT and pH 7.0 and negative potential on the opposite

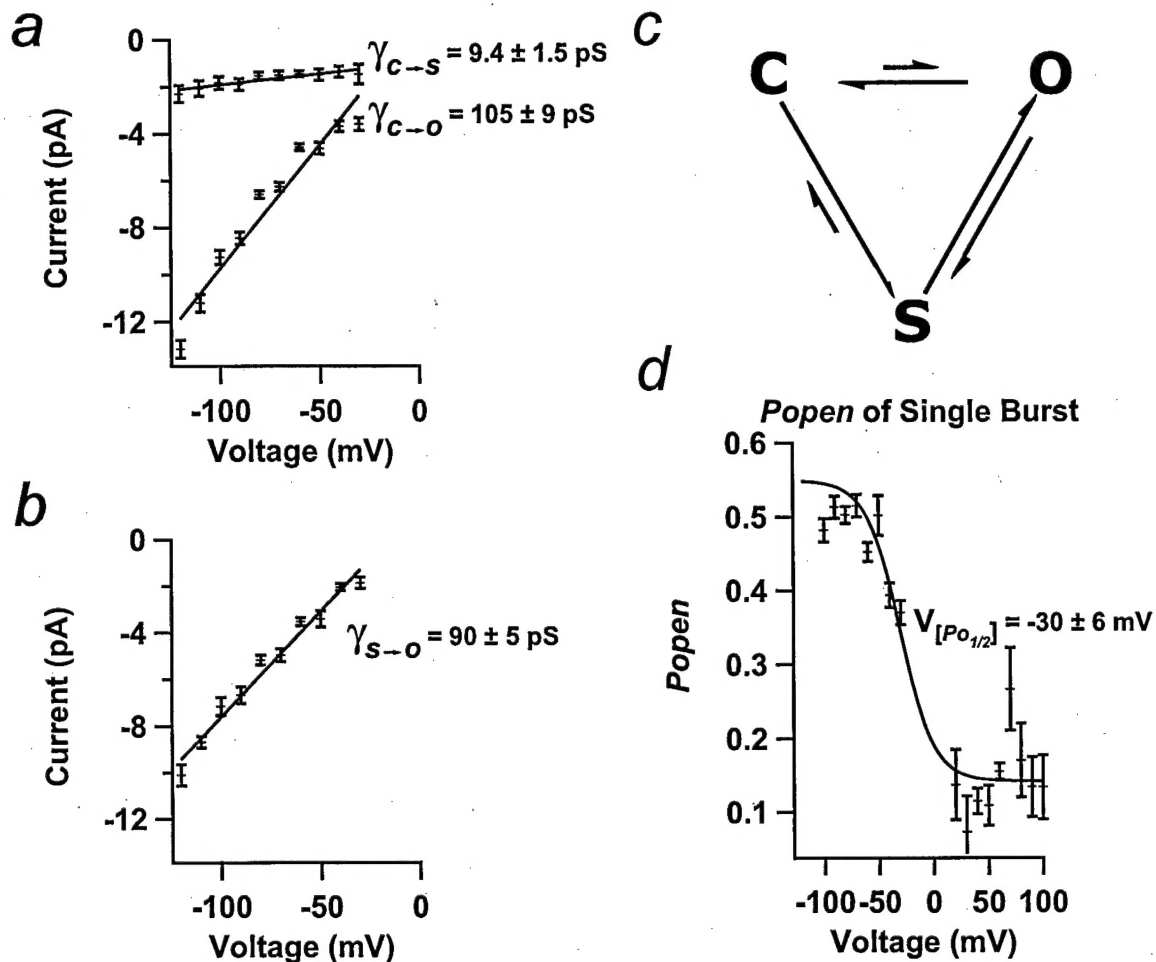
compartment. This suggests that the BoNT heavy chain channel would be closed in the endosome until it is gated by the BoNT light chain to initiate its translocation across the membrane into the cytosol. The neuroblastoma cell line appears, therefore, to be a suitable system to characterize the BoNT channel and to pursue evaluation of plausible strategies for targeted drug delivery thereby minimizing the requirement for *in vivo* animal testing.

#### References

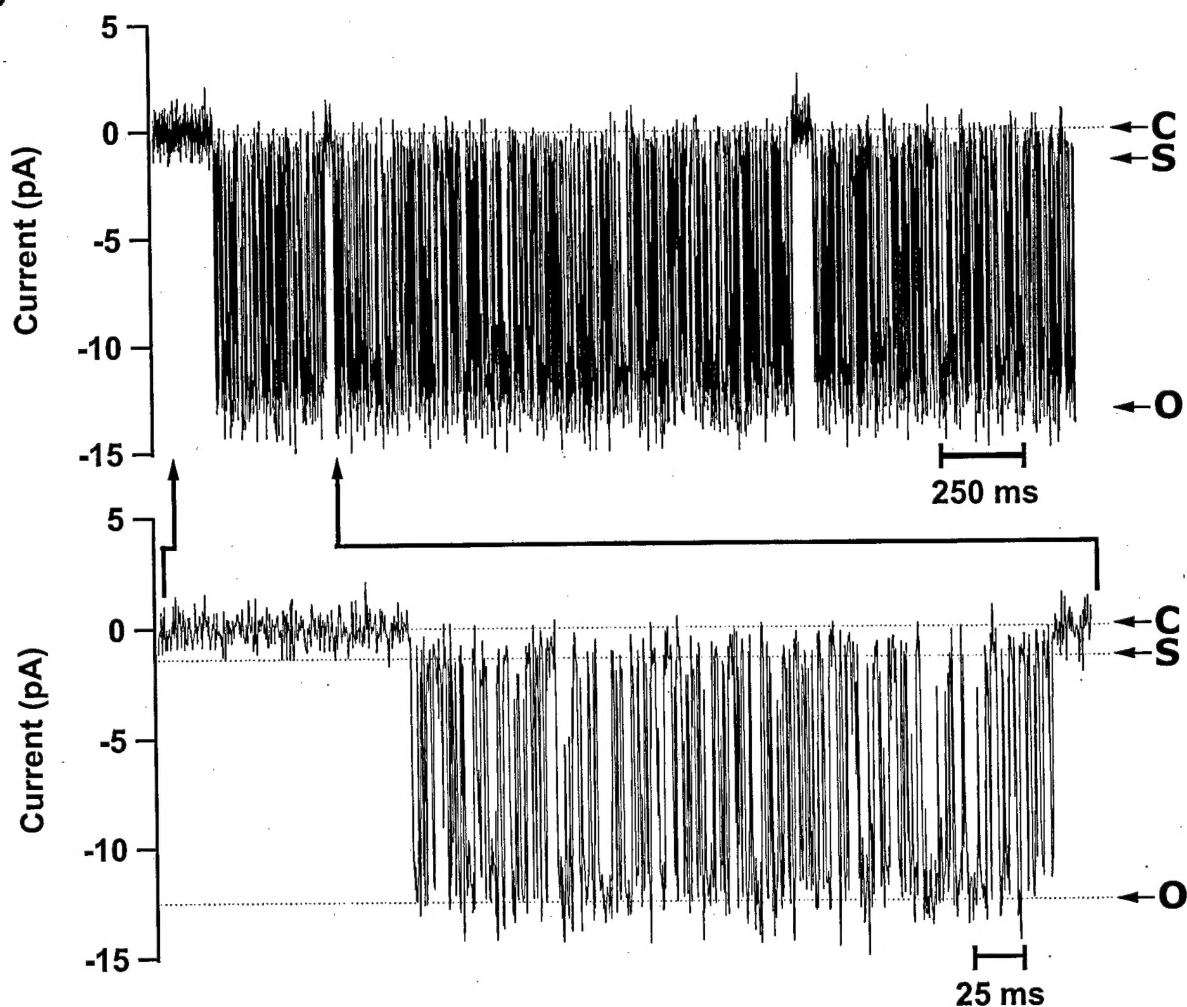
1. Koriazova, L. K. & Montal, M. Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. *Nat Struct Biol* **10**, 13-8 (2003).
2. Schiavo, G., Matteoli, M. & Montecucco, C. Neurotoxins affecting neuroexocytosis. *Physiol Rev* **80**, 717-66. (2000).
3. Yowler, B. C., Kensinger, R. D. & Schengrund, C. L. Botulinum neurotoxin A activity is dependent upon the presence of specific gangliosides in neuroblastoma cells expressing synaptotagmin I. *J Biol Chem* **277**, 32815-9 (2002).
4. Hille, B. *Ion Channels of Excitable Cells* (Sinauer, Sunderland, MA, 2001).
5. Sheridan, R. E. Gating and permeability of ion channels produced by botulinum toxin types A and E in PC12 cell membranes. *Toxicon* **36**, 703-17. (1998).
6. Keller, B. U., Hartshorne, R. P., Talvenheimo, J. A., Catterall, W. A. & Montal, M. Sodium channels in planar lipid bilayers. Channel gating kinetics of purified sodium channels modified by batrachotoxin. *J Gen Physiol* **88**, 1-23. (1986).
7. Gambale, F. & Montal, M. Characterization of the channel properties of tetanus toxin in planar lipid bilayers. *Biophys J* **53**, 771-83. (1988).



**Figure 1.** Botulinum neurotoxin bursting channel activity in excised patches of Neuro 2A cells. Representative single-channel currents at the indicated voltages. Consecutive stimulations on the same patch. Channel opening is indicated by a downward deflection. BoNT A channels open to a main conductance of  $\sim 90$  pS.



**Figure 2.** Analysis of BoNT A channels in Neuro 2A cells. a, Single channel current-voltage characteristics for transitions of the closed state (C) to substate (S) and closed state to open state (O). b, Single channel current-voltage characteristics for transitions of the substate to the open state. c, Transitions between a closed (C), substate (S) and fully open (O) state of BoNT A channels. Arrow length is proportional to probability of transition. d, Probability of channel residence in the open state as a function of voltage. All analysis based upon single bursts of channel activity within a record ( $N \geq 6$ ).



**Figure 3.** BoNT A bursting channel activity in excised patches of Neuro 2A cells. At -130 mV transitions from the closed (C) to the open (O) state and from the substate (S) to the open state are clearly resolved. The BoNT A channels open to a main conductance of ~90 pS and a subconductance of ~10 pS. The approximate value for the subconductance state (S) is marked. The lower panel shows the section of the record delimited by the arrows at higher time resolution.